Lasers in Surgery and Medicine 19:201–206 (1996)

Reactive Oxygen Species Inducible by Low-Intensity Laser Irradiation Alter DNA Synthesis in the Haemopoietic Cell Line U937

Gary A. Callaghan, BSc, Cathal Riordan, BSc, William S. Gilmore, PhD, Irene A. McIntyre, MSc, James M. Allen, PhD, and Bernadette M. Hannigan, PhD

Biotherapeutics Research Group, School of Biomedical Sciences, University of Ulster at Jordanstown (BT37 0QB) (G.A.C., C.R., I.A.M.) and Coleraine (BT52 1SA) (W.S.G., J.M.A., B.M.H.), Northern Ireland

Background and Objective: **The purpose of this study was to evaluate the possible role of reactive oxygen species (ROS) in mediating previously recorded alterations in DNA synthesis, inducible by low-intensity laser irradiation (LILI), in the haemopoietic cell line U937.**

Study Design/Materials and Methods: **The ability of LILI (660 nm, 12 mW, 5 kHz) to induce ROS from U937 cells was assessed spectrophotometrically at energy densities (E.D.) from 1.0 to 11.5 J/cm^2. In order to assess whether laser-induced ROS could alter cellular proliferation DNA synthesis was measured, post-irradiation, by the incorporation of tritiated thymidine (^{3}H-TdR) into the cells in both the presence and absence of the antioxidant catalase (CAT).**

Results: **Detectable ROS were produced post-irradiation only from the differentiated form of the cell line. Analysis by Student's *t*-test for unrelated groups showed a significant difference, at E.D.s 2.9 and 8.6 J/cm^2, in the extent of DNA synthesis occurring in cells irradiated in the presence of CAT or in its absence.**

Conclusion: **These findings demonstrate that laser-inducible ROS can mediate laser's effects on this cell line.**

© 1996 Wiley-Liss, Inc.

Key words: DNA synthesis, macrophage, antioxidant

INTRODUCTION

Over the last 20 years low-intensity laser irradiation (LILI) has become established as a successful method of promoting tissue repair [1,2]. Despite many reports of this success, the biological basis of the laser's efficacy remains ill defined [3].

Research conducted from various perspectives is striving to reveal the precise biological process behind the success of laser irradiation, for example, the effect of laser irradiation on oxidative metabolism of cells as evaluated by chemiluminesence [4,5] measurements and the ability of laser light–stimulated cells to incorporate tritiated thymidine (^{3}H-TdR) [6, 7].

This study examined the effect of LILI on the production of reactive oxygen species (ROS) by the human haemopoietic cell line U937 and the possibility that such ROS could mediate previously documentated effects of LILI on these cells [6]. To date investigation of ROS production following LILI has been limited [2]. The U937 cell line provides an efficient in vitro model of either monocytic or granulocytic cells, depending on experimental conditions, and as such can represent

Accepted for publication August 22, 1995.

Address reprint requests to Dr. B.M. Hannigan, Biotherapeutics Research Group, School of Biomedical Sciences, University of Ulster, Coleraine, BT52 1SA, Northern Ireland.

© 1996 Wiley-Liss, Inc.

essential components of the cellular phase of the in vivo wound healing process [8].

The initial aim of the study was to confirm increased ROS production by U937 cells following LILI. Results obtained from microassays of the superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) enabled calculation of the amounts of the antioxidant catalase (CAT) required to neutralise any ROS produced. Catalase, which removes H_2O_2, was then used to identify how the effects of LILI are altered by the absence of ROS.

In previous experiments carried out in this laboratory [6] it was demonstrated that LILI, at energy densities above an apparent threshold dose, induced a decrease in ^{3}H-TdR incorporation. In the current study the ability of the cells to incorporate ^{3}H-TdR following irradiation was used to indicate the level of LILI-induced de novo DNA synthesis [9,10] in the presence or absence of appropriate amounts of CAT. The observed patterns of LILI-induced ^{3}H-TdR incorporation would then allow a conclusion to be reached on whether or not ROS have any part to play in laser-induced cell proliferation.

MATERIALS AND METHODS

Cell Culture Conditions

The human haemopoietic cell line U937 was maintained in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated foetal calf serum and 1% (v/v) penicillin/streptomycin (pen/strep). Sub-culturing was approximately every 2–3 days for undifferentiated U937 cells. The cells were grown in 75-cm^2 Falcon sterile culture flasks and incubated at 37°C in a humidified atmosphere containing 5% CO_2 in air. For certain experiments the U937 cell line was exposed to dimethyl sulphoxide (DMSO; Sigma) 1.25% (v/v) for 3 days to induce cell differentiation.

Preparation of Cells for Assay

Initial experiments showed that the U937 cell line had an exponential pattern of growth at cell densities between 2×10^5 and 1×10^6 cells/ml. Therefore, in the experiments reported here, the cells were allowed to reach a concentration of 4×10^5 cells/ml to ensure they were at an actively growing stage. Cell suspensions were centrifuged (750*g* for 5min) and resuspended at 5×10^6 cells/ml in phosphate-buffered saline (PBS) supplemented with 2 mM $CaCl_2$ and 4 mM glucose.

The U937 cell line, in both its differentiated and undifferentiated forms, was then assayed for its ability to produce the ROS O_2^- and H_2O_2 following LILI at a range of energy densities (E.D.).

Irradiation Parameters

The laser used was a Biotherapy 3ML (Omega Universal Technologies, London). This laser contains an active GaA1As medium (660 nm, 12 mW, pulsed at 5 kHz). The area of the spot size with the active head positioned 1 cm above cell suspension was 0.125 cm^2. The irradiance was calculated as 0.096 W/cm^2 Using the equation,

$$\text{Time (s)} = \frac{\text{Energy Density (J/cm}^2\text{)}}{\text{Irradiance (W/cm}^2\text{)}}$$

irradiation times for energy densities of 1.0, 2.9, 5.8, 8.6, 11.5, and 14.4J/cm^2 were calculated [11].

Irradiation of Cells

One hundred-millilitre aliquots of cells were distributed in a 96-well microtitre plate. To minimise cross-irradiation between wells, at least two empty wells separated the experimental (irradiated) wells. Plates were returned to the incubator until ready for use, no more than 1 h later. Plates were shaken immediately prior to irradiation to maintain cells in suspension. During irradiation, plates were put on a matte black surface and the laser probe was positioned exactly 1 cm above the surface of the cell suspension using a specially designed collar. Four wells were irradiated at each energy density in each experiment.

Superoxide Anion Production

Following laser irradiation O_2^- production was quantified by the modified method of Pick [12]. The assay is based on the reduction of ferricytochrome C by O_2^- read as absorbance at 550 nm using a microtitre plate reader (Dynatech, England). Readings were recorded at 15-min intervals over a 90-min period. Between readings the plates were incubated at 37°C in 5%CO_2 in humidified air. The absorbance values were converted to nanomoles of O_2^-/well using Beers Law with an extinction coefficient for cytochrome C of 21×10^3 M^{-1} cm^{-1}.

Hydrogen Peroxide Production

Following irradiation, H_2O_2 production was quantified by the modified method of Pick and Keisari [13]. This assay is based on the horseradish peroxidase (HRPO) dependent oxidation of phenol red by H_2O_2. This leads to the formation of

a compound that, at alkaline pH, exhibits increased absorbance at 600 nm.

Readings were recorded at 15-min intervals over a 90-min period. Between readings the plates were incubated at 37°Cin 5% CO_2 in humidified air. Ten microlitres of 1 M NaOH was added to each well 2 min before reading absorbance values at 600 nm. The absorbance values were converted to nanomoles of H_2O_2/well using standard curves based on serial dilutions of H_2O_2 between 0.1 and 5 nM.

Neutralising ROS by Addition of Antioxidants

An amount of the antioxidant (CAT) in excess of that required to neutralise the H_2O_2 produced was estimated from the previous two assays.

Cell suspensions were centrifuged (750*g*, 5 min) and resuspended in RPMI 1640 and 1% pen/strep at 1×10^6 cells/ml. Foetal calf serum was not included in the medium to exclude the presence of exogenous growth stimulating factors. Controls used were unirradiated cells suspended in RPMI 1640. The cells were added to microtitre plates and irradiated at the same E.D. range used previously.

Tritiated Thymidine Incorporation

Following irradiation, cells were incubated for 24 h (37°C, 5% CO_2 in air), and ^{3}H-TdR incorporation was determined by adding 1 μCi of ^{3}H-TdR to each well for a further 4 h following incubation. (This technique allows DNA synthesis to be assessed by measuring the incorporation of the acid-soluble tritiated thymidine into an acid-insoluble form when incorporated in DNA [9,10]). The samples were then harvested onto nitrocellulose filters using a Canberra Packard Micromate 196 Cell Harvester. The discs were allowed to dry and removed to vials containing 4 ml Optiphase 'HiSafe' II scintillation fluid. Radioactivity, expressed as counts per minute (cpm $\times 10^3$), was assessed using an LKB 1214 Rackbeta Liquid Scintillation Counter.

Cell Viability and Temperature

Cell viability was assessed 24 h after irradiation using the Trypan Blue exclusion test. In all experiments reported here, the viability of the cells remained >95% following the 24 h incubation period.

To test for any thermal effects of irradiation, the temperature of the cell suspension in the well was recorded at each energy density during and after irradiation using a specially adapted thermistor probe. The probe was attached to a squirrel data logger (1200 series, Grant Instruments, England) connected to an IBM compatible personal computer (Amstrad, England). This probe was capable of detecting temperature changes within the range −30°C to +65°C, accurate to 0.05°C. In all the experiments no significant temperature change ($P > .05$) was observed during or after irradiation.

Statistical Analysis of Results

Results were analysed using unrelated one-way analysis of variance comparing the individual doses and the appropriate control. Corresponding doses applied under different circumstances were analysed using unrelated Student's *t*-test.

RESULTS

O_2^- Production

Undifferentiated U937 cells did not produce detectable amounts of O_2^- post-irradiation whereas DMSO-differentiated cells showed a marked increase in O_2^- production relative to the unirradiated control. O_2^- production reached a peak 45 min post-irradiation, with the lower E.D.s (1.0 and 2.9 J/cm^2) showing the greatest increase. Figure 1 summarises the response in all experiments (n = 3), of differentiated U937 cells, and illustrates nanomoles of O_2^- per 1×10^5 cells produced at various time periods for a range of energy densities. Standard errors were calculated for the mean absorbances before the results were converted to nmol $O_2^-/10^5$ cells. The standard errors were within 10% of the mean.

H_2O_2 Production

As with O_2^- the undifferentiated cells did not produce detectable amounts H_2O_2 after laser stimulation. There was a marked increase in H_2O_2 production, as compared to the control, in the differentiated cells post-irradiation. Unlike O_2^- production, H_2O_2 was detectable almost instantaneously post-irradiation and continued to be detectable throughout the time period of the assay. H_2O_2 production was similar at all E.D.s. Figure 2 summarises the response of differentiated U937 cells in their ability to produce H_2O_2 after laser stimulation. The results are expressed as nanomoles of H_2O_2 per 1×10^5 cells at the same time periods and range of energy densities as in Figure 1.

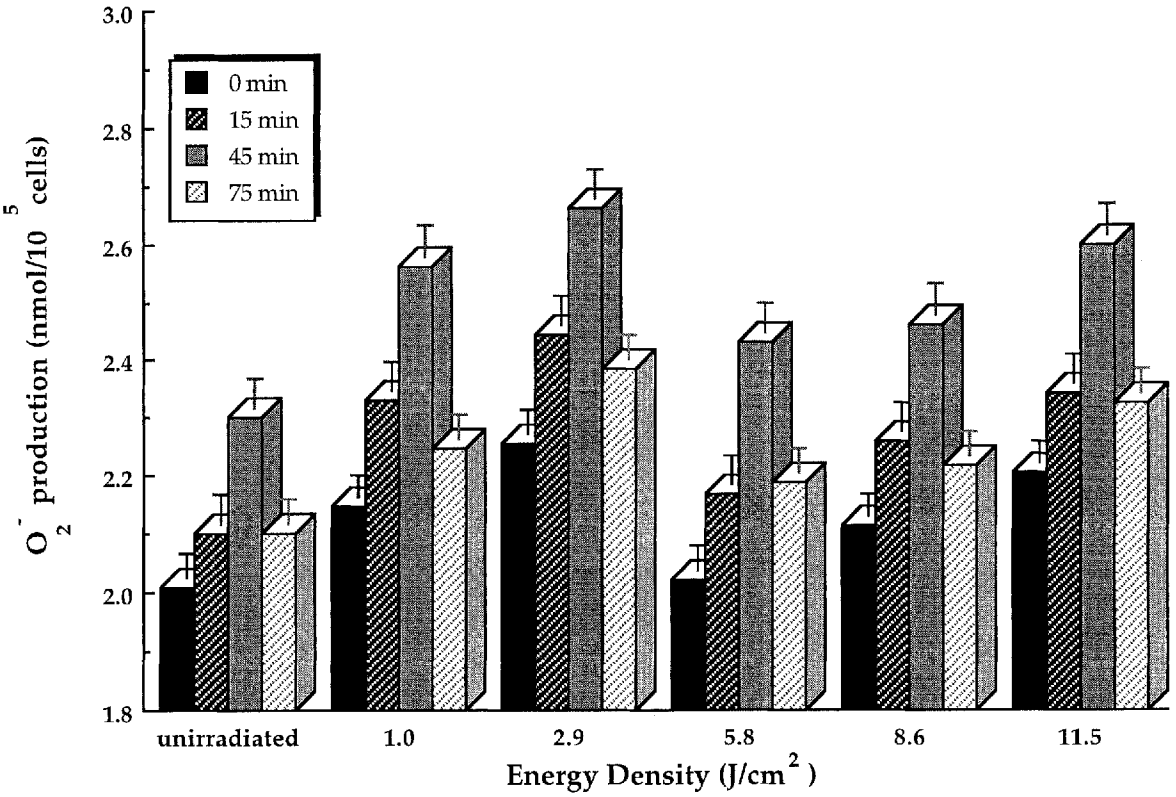

Fig. 1. The graph shows the amount of O_2^- produced (in nmol/10^5 cells) from the U937 cell line at various time periods following laser irradiation at E.D.s between 1.0 and 11.5 J/cm^2. The values are the mean of 12 observations (from three independent experiments), and vertical lines show 1 s.e.m.

^{3}H-TdR Incorporation

Irradiation of the cell line at E.D.s between 2.9 and 11.6 J/cm^2 caused a reduction in labelled base incorporation of between 5% and 7%. Statistical analysis of the raw data showed that the change in ^{3}H-TdR incorporation was significant at 5.8 and 8.6 J/cm^2 ($P < .05$) as compared to the control (unirradiated cells). Figure 3 summarises the proliferative response of cells (n = 3 independent experiments) and plots mean ^{3}H-TdR incorporation as the percentage change from the control (unirradiated) cells. Vertical lines are ±1 standard error of the mean (s.e.m.).

The addition of CAT immediately prior to irradiation caused a reversal of the previously recorded reduction in labelled base incorporation shown in Figure 3. Statistical analysis showed that, at E.D. 2.9 and 8.6 J/cm^2, the presence of CAT significantly ($P < .05$) changed the LILI-induced inhibition of ^{3}H-TdR incorporation noted in Figure 3. Figure 4 plots a summary of the response for experiments (n = 3) carried out in parallel with those shown in Figure 3, in which CAT (60 Sigma units/ml) was added immediately prior to irradiation. The mean change in ^{3}H-TdR incorporation is again represented as a percentage change relative to the control value.

DISCUSSION

The initial aim of this investigation was to quantify LILI-induced ROS production from the U937 cell line. Both differentiated and undifferentiated cells of this line were irradiated (660 nm, 5 kHz, 0.012 W) at a range of E.D.s within the therapeutic range (1.0–11.5 J/cm^2) and O_2^- and H_2O_2 production measured spectrophotometrically. The findings showed that LILI could induce detectable amounts of ROS only from differentiated cells. These results parallel previously reported studies [8] where only the differentiated form of the cell line could produce ROS in response to a variety of known ROS inducers.

Once it had been established that ROS were

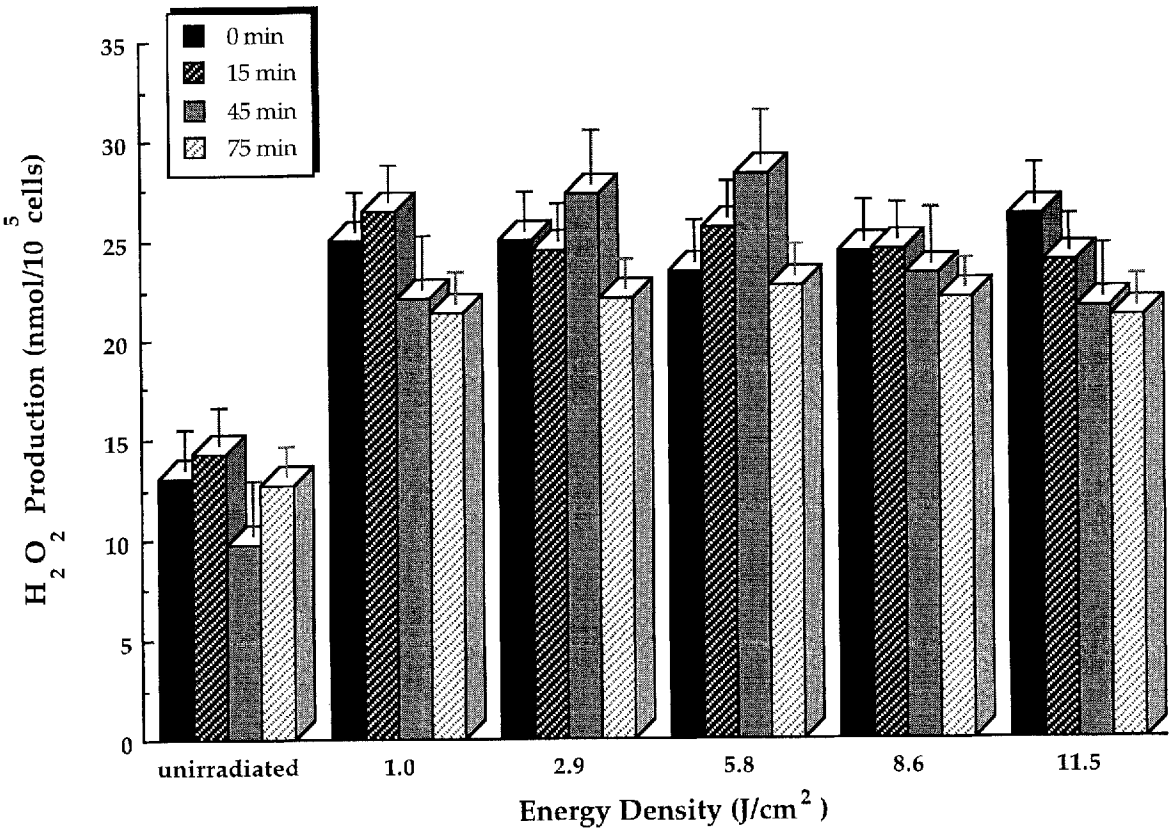

Fig. 2. The graph shows the amount of H_2O_2 produced (in nmol/10^5 cells) from the U937 cell line at various time periods following laser irradiation at E.D.s between 1.0 and 11.5 J/cm^2. The values are the mean of four observations (from two independent experiments), and vertical lines show 1 s.e.m.

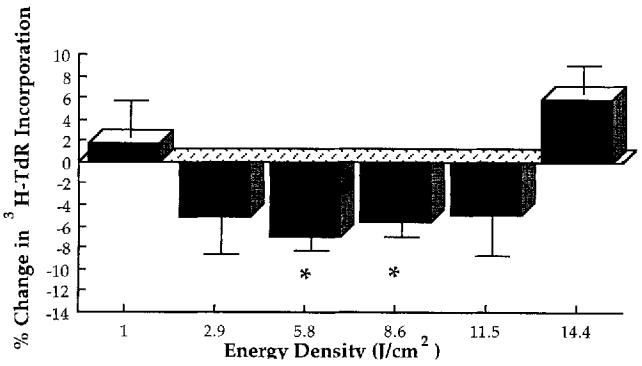

Fig. 3. The graph shows the percentage change from the control in ^{3}H-TdR incorporation into the U937 cell line 24 h post-irradiation at E.D.s between 1.0 and 14.4 J/cm^2. The bars are the mean values of 12 observations (from three independent experiments), and vertical lines show 1 s.e.m. Significant differences from the unirradiated control are indicated (*$P < .05$).

inducible by LILI, the potential role of ROS in mediating previously reported laser-inducible changes in DNA synthesis in the U937 cell line was evaluated [6]. The findings showed that the presence of CAT significantly changed ($P < .05$) ^{3}H-TdR incorporation into U937 cells. The action of CAT is to remove H_2O_2. These results suggest that the removal of LILI-induced ROS has a direct effect in negating the previously reported effect of LILI on DNA synthesis.

As mammalian cells exist in a constant oxidative siege requiring an appropriate balance of oxidants and antioxidants [14], inducement of either can modulate biological processes [15]. In such cells, three unrelated transcription factor systems, NF-κB, AP-1, and SRF/TCF, have been identified as regulators of transcription in response to alterations of the intracellular level of ROS [16].

Catalase is unlikely to enter the cells to which it is exposed in this system. Of the ROS which were measured, O_2^- and H_2O_2, the latter is highly likely to cross cell membranes [15]. The activity of CAT in removing H_2O_2 would then be facilitated and occur outside the cell. Thus it is these ROS, produced within the laser-irradiated cells and released to the surrounding medium,

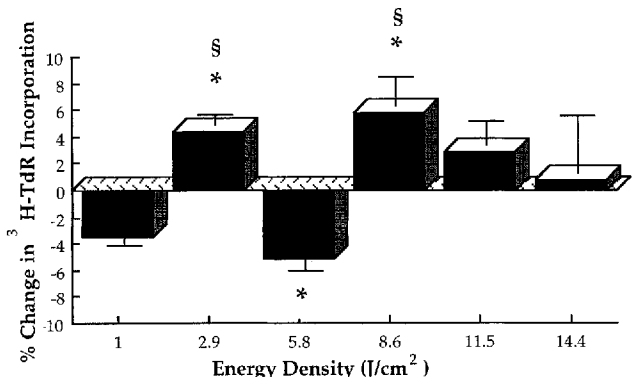

Fig. 4. The graph shows the percentage change from the control in ^{3}H-TdR incorporation into the U937 cell line 24 h post-irradiation, in the presence of catalase (60 Sigma units/ml) at E.D.s between 1.0 and 14.4 J/cm^2. The bars are the mean values of 12 observations (from three independent experiments), and vertical lines show 1 s.e.m. Significant differences from the unirradiated control (*$P < .05$) and significant differences from corresponding values noted in Figure 3 are indicated (§ $P < .05$).

which appear to have mediated the altered DNA synthesis. Further experiments are exploring the effect of smaller molecules with antioxidant activities which can penetrate into cells. A comparison of those new data with the results reported in this paper may give a better understanding of how antioxidants can interfere with laser-mediated events.

In conclusion, whatever transcription factor system may be involved, we have shown that LILI does induce ROS in the U937 cell line, which when removed negates the significant changes in the cells' DNA synthesis inducible by laser.

ACKNOWLEDGMENTS

This work was supported by grants from the Department of Health and Social Services for Northern Ireland and the Physiotherapy Research Foundation.

REFERENCES

1. Mester E, Mester AF, Mester A. The biological effect of laser application. Lasers Surg Med 1985; 5:31–39.
2. Kitchen SS, Partridge CJ. A review of low laser therapy. Physiotherapy 1991; 77(3):161–168.
3. Karu T. Photobiological fundamentals of low power laser therapy. J Quantum Electronics 1987;23:1703–1715.
4. Shiroto C, Sugawara K, Kumae T, Ono Y, Sasaki M, Ohshiro T. Effect of diode laser radiation *in vitro* on activity of human neutrophils. Low Level Laser Therapy 1989; 3:135–140.
5. Karu TI, Ryabykh TP, Fedoseyeva GE, Puchkova NI. Helium-Neon laser-induced respiratory burst of phagocytic cells. Lasers Surg Med 1989; 9:585–588.
6. O'Kane S, Shields TD, Gilmore WS, Allen JM. Low intensity laser irradiation inhibits tritiated thymidine incorporation in the hemopoietic cell lines HL-60 and U937. Lasers Surg Med 1994; 14:34–39.
7. Agaiby A, Dyson M. The effects of light on T-lymphocytes proliferation in vitro. Lasers Surg Med Suppl 1992; 4:40.
8. Harris P, Ralph P. Human leukaemic models of myelomonocytic development: a review of the HL-60 and U937 cell lines. J Leukoc Biol 1985; 37:407–422.
9. Bodycote Y, Wolff S. Metabolic breakdown of [H-3] thymidine and the inability to measure human lymphocyte proliferation by incorporation of radio activity. Proc Natl Acad Sci USA 1986; 83:4749–4753.
10. Oldham KG. Problems in radiometric assessment of protine and nucleic acid synthesis. Anal Biochem 1971; 44:143–153.
11. Calderhead RG, On the correct reporting of parameters and consistent use of terminology in reporting clinical and experimental LLLT procedures. Laser Therapy 1990; 3:2.
12. Pick E. Microassays for superoxide and hydrogen peroxide production and nitroblue tetrazolium reduction using an enzyme immunoassay microplate reader. In: DiSabato G, Everse J, eds. "Methods in Enzymology." London: Academic Press, 1986, vol 132, pp 402–421.
13. Pick E, Keisari, Y. A simple colourimetric method for the measurement of hydrogen peroxide produced by cells in culture. J Immunol Methods 1980; 38:161–165.
14. Buttke TM, Sandstorm PA. Oxidative stress as a mediator of apoptosis. Immunol Today 1994; 15:7–10.
15. Hunt NH, van Reyk DM, Fragonas JC, Jeitner TM, Goldstone SD. Redox mechanisms in T cell activation. In: Pasquier C, eds. "Oxidative Stress, Activation and Viral Infection." Basel, Switzerland: Birkhäuser Verlag, 1994, pp 237–251.
16. Meyer M, Schreck R, MÜller JM, Baeuerle PA. Redox control of gene expression by eukaryotic transcription factors NF-κB, AP-1 and SRF/TCF. In: Pasquier C, eds. "Oxidative Stress, Activation and Viral Infection." Basel, Switzerland: Birkhäuser Verlag, 1994, pp 217–235.